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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/381,747	09/22/1999	MAR TORMO	UTSC:550---/1PAR	4363

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EXAMINER	
CHONG, KIMBERLY	
ART UNIT	**PAPER NUMBER**
1635	

DATE MAILED: 11/15/2005

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No. 09/381,747	Applicant(s) TORMO ET AL.	
	Examiner Kimberly Chong	Art Unit 1635	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE ____ MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *19 August 2005*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-20 and 22-49* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-20 and 22-49* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: _____.

DETAILED ACTION

Status of Application/Amendment/Claims

Applicant's response filed 08/19/2005 has been considered. Rejections and/or objections not reiterated from the previous office action mailed 05/17/2005 are hereby withdrawn. The following rejections and/or objections are either newly applied or are reiterated and are the only rejections and/or objections presently applied to the instant application.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

With entry of the amendment filed on 08/19/2005, claims 1-20 and 22-49 are pending in the application.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-20 and 22-38 are provisionally rejected under the judicially created doctrine of double patenting over claims 58-65, 72-76, 79-89, 91 and 92 of copending Application No. 08/726,211. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the copending application are drawn to patentably indistinguishable subject matter

The instant claims are drawn to a composition comprising a P-ethoxy polynucleotide that hybridizes to a Bcl-2-encodng polynucleotide to form a neutrally-charged polynucleotide/lipid association wherein the polynucleotide is between 8-50 bases, the P-ethoxy is complementary to the translation initiation site of Bcl-2 mRNA, the polynucleotide is an oligonucleotide comprising SEQ ID NO.1, or wherein said neutral lipid is a phosphatidycholine, phosphatidylglycerol, phosphatidylethanolamine and further the composition comprises an expression cassette that encodes a P-ethoxy polynucleotide. The subject matter of the instant claims is further drawn to a method of inhibiting a Bcl-2 associated disease in a cancer comprising administering a polynucleotide/lipid association that hybridizes to a Bcl-2 encoding polynucleotide wherein the association comprising an oligonucleotide 8-50 bases, the association is delivered in a volume of 0.50-10.0 ml per dose, the association is delivered in an amount from about 5-30 mg per m2 and the antisense is from 8-50 bases and is a P-ethoxy polynucleotide.

Claims 58-65, 72-76, 79-89, 91 and 92 of copending Application No. 08/726,211 are drawn to a composition comprising a polynucleotide that hybridizes to a Bcl-2-encodng polynucleotide to form a neutrally-charged polynucleotide/lipid association wherein the polynucleotide is between 8-50 bases, the polynucleotide is complementary to the translation initiation site of Bcl-2 mRNA, the polynucleotide is a P-ethoxy oligonucleotide, the

polynucleotide is an oligonucleotide comprising SEQ ID NO.1, or wherein said neutral lipid is a phosphatidycholine, phosphatidylglycerol, phosphatidylethanolamine and further the composition comprises an expression cassette that encodes a P-ethoxy polynucleotide.

The method of the instant claims are encompassed in the methods of claims 11-15, 18-20, 22-25, 28-30, 44-46, 58-65, 72-76, 79-89, 91 and 92 of copending Application No. 08/726,211 because disease cells having a t(14;18) translocation are cancer cells as evidence by Korsmeyer et al. (Cancer Biology 1993) who states a t(14;18) translocation constitutes the most common chromosomal translocation in human lymphoid malignancies (see page 328).

This is a provisional double patenting rejection since the conflicting claims have not yet been patented.

Claims 39-49 are provisionally rejected under the judicially created doctrine of double patenting over claims 11-15, 18-20, 22-25, 28-30 and 44,46 of copending Application No. 08/726,211. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims and the claims of the copending application are drawn to patentably indistinguishable subject matter

The subject matter of the instant claims are drawn to a method of inhibiting a Bcl-2 associated disease in a cell that expresses both Bcl-2 and Bax and comprises administering a polynucleotide/lipid association that hybridizes to a Bcl-2 encoding polynucleotide wherein the association comprising an oligonucleotide 8-50 bases, the association is delivered in a volume of 0.50-10.0 ml per dose, the association is delivered in an amount from about 5-30 mg per m2 and the antisense is from 8-50 bases.

The subject matter of the claims 11-15, 18-20, 22-25, 28-30, 44-46 of copending Application No. 08/726,211 are drawn to a method of inhibiting a Bcl-2 associated disease in a cell, wherein the cells have a t(14;18) translocation and wherein the method comprises administering a polynucleotide/lipid association that hybridizes to a Bcl-2 encoding polynucleotide wherein the association comprising an oligonucleotide 8-50 bases, the association is delivered in a volume of 0.50-10.0 ml per dose, the association is delivered in an amount from about 5-30 mg per m2 and the antisense is from 8-50 bases.

The method of the instant claims are encompassed in the methods of claims 11-15, 18-20, 22-25, 28-30, 44-46 of copending Application No. 08/726,211 because as evidenced by Korsmeyer et al. (Cancer Biology 1993) a cell comprises both Bcl-2 and Bax which are essential in determining the survival or death of cells following an apoptotic stimulus (see Abstract and page 331).

Response to Applicant's Arguments

Correction of Filing Receipt

It is noted that Applicants are requesting correcting of filing receipt. The request has been forwarded to the appropriate department.

Claim Rejections - 35 USC § 112

The rejection of claims 10-20, 25 and 39-49 under 35 U.S.C. 112, first paragraph enablement, in the Office Action mailed 02/01/2001 has been withdrawn in response to Applicant's arguments.

The rejection of claims 1-9, 21-24 and 26-38 as being obvious under 35 U.S.C. 103(a) over Evan (WO 93/20200) or Reed (WO 95/08350) or Green et al. (U.S. Patent No. 5, 583,034) each in view of Lopez-Berestein et al. (U.S. Patent No. 5, 855,911) has been overcome due to Applicant's statement of common obligation of assignment between Lopez-Berestein et al. (U.S. Patent No. 5, 855,911) and the instant application.

MPEP 706.02(l)(2) II states "...if the applicant(s) or an attorney or agent of record makes a statement to the effect that the application and the reference were, at the time the invention was made, owned by, or subject to an obligation of assignment, the same person.", this statement is sufficient evidence to establish common ownership at the time the invention was made.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Kimberly Chong whose telephone number is 571-272-3111. The examiner can normally be reached Monday thru Friday between 7-4 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Andrew Wang can be reached at 571-272-0811. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is

(866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public. For more information about the PAIR system, see http://pair-direct.uspto.gov.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

Kimberly Chong
Examiner
Art Unit 1635

SEAN McGARRY
PRIMARY EXAMINER
1635

Notice of References Cited	Application/Control No. 09/381,747	Applicant(s)/Patent Under Reexamination TORMO ET AL.	
	Examiner Kimberly Chong	Art Unit 1635	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
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	R					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Korsmeyer et al. Bcl-2?Bax: a rheostat that regulates an anti-oxidant pathway and cell death. Cancer Biology, 1993, Vol. 4: 327-332. Academic Press Ltd.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

seminars in **CANCER BIOLOGY**, Vol 4, 1993 : pp 327-332

Bcl-2/Bax: a rheostat that regulates an anti-oxidant pathway and cell death

Stanley J. Korsmeyer, John R. Shutter, Deborah J. Veis, Diane E. Merry and Zoltan N. Oltvai

The maintenance of homeostasis in normal tissues reflects a balance between cell proliferation and cell death. The importance of both positive and negative regulators of cell growth has been well documented in neoplasia. Bcl-2 argues for the existence of a new category of oncogenes, regulators of cell death. The bcl-2 gene was identified at the chromosomal breakpoint of t(14;18) bearing B cell lymphomas. Bcl-2 has proved to be unique among protooncogenes in blocking programmed cell death rather than promoting proliferation. In adults, bcl-2 is topographically restricted to progenitor cells and longlived cells but is much more widespread in the developing embryo. Transgenic mice that overexpress bcl-2 in the B cell lineage demonstrate extended cell survival, and progress to high grade lymphomas. Bcl-2 has been localized to mitochondria, endoplasmic reticulum and nuclear membranes, also the sites of reactive oxygen species generation. Bcl-2 does not appear to influence the generation of oxygen free radicals but does prevent oxidative damage to cellular constituents including lipid membranes. Bcl-2 deficient mice complete embryonic development and display relatively normal haematopoietic differentiation but undergo fulminant lymphoid apoptosis of thymus and spleen. Moreover, they demonstrate two potentially oxidation related pathologies: polycystic kidney disease and hair hypopigmentation. A family of bcl-2 related genes is emerging that includes Bax, a conserved homolog that heterodimerizes in vivo with bcl-2. A pre-set ratio of Bcl-2/Bax appears to determine the survival or death of cells following an apoptotic stimulus.

Key words: apoptosis / cell death / Bcl-2 / oncogene / free radical

Bcl-2 initiates a new category of oncogenes: regulators of cell death

Malignancies usually possess aberrations in more than a single pathway.[1] Either increased proliferation or decreased death might result in an expansion of cell numbers (Figure 1). To date, most of our knowledge concerning oncogenic events has concentrated upon mechanisms of increased growth and proliferation. Studies of bcl-2 emphasize the existence of multiple pathways in the generation of neoplasia (Figure 1). The increased cell number in neoplastic tissue can be viewed as a violation of normal homeostasis. The maintenance of homeostasis in normal tissue, in many respects, reflects a simple balanced equation of input (cellular proliferation and renewal) versus output (cell death). The maintenance of remarkably invariant cell numbers must reflect tightly regulated death pathways as well as controlled proliferation (Figure 1).

Programmed cell death represents a cell autonomous suicide pathway that helps restrict cell numbers. The well defined loss of specific cells is crucial during embryonic development as part of organogenesis.[2] In mature tissues, genetically programmed demise regulates the volume of cells. Andrew Wyllie identified a morphologically distinct and highly ritualistic cell death entitled apoptosis.[3] Cells dying by apoptosis display marked plasma membrane blebbing, volume contraction, nuclear condensation and the activation of an endonuclease that cleaves

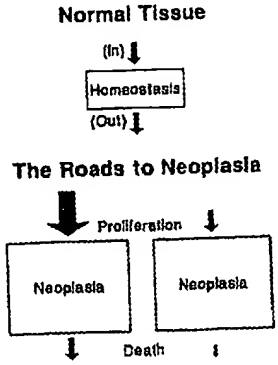

Figure 1. Schematic representation of normal tissue homeostasis with balanced input and output reactions. Alternative roads to neoplasia are depicted as either increased proliferation (In) or decreased death (Out).

From the Howard Hughes Medical Institute at Washington University School of Medicine, 660 South Euclid Avenue, St Louis, MO 63110, USA

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DNA into nucleosomal length fragments. Defining the precise genes and biochemical events that regulate this death program holds the promise for novel therapeutic approaches.

The t(14;18) breakpoint of follicular lymphoma reveals the bcl-2 gene

The t(14;18)(q32;q21) constitutes the most common chromosomal translocation in human lymphoid malignancies. Approximately 85% of follicular and 20% of diffuse B cell lymphomas possess this translocation.[4,5] As a disease, follicular lymphoma provided many clues concerning the ultimate function of the bcl-2 molecule. Follicular lymphoma often presents as a low-grade malignancy comprised of small resting IgM/IgD B cells. Over time conversion to an aggressive high grade lymphoma with a diffuse large cell architecture frequently occurs in these patients.[6] The location of the immunoglobulin (Ig) heavy chain locus at 14q32 and the B cell phenotype of this lymphoma provided the rationale for cloning the chromosomal breakpoint. Aberrant Ig heavy chain gene rearrangements in t(14;18) lymphomas proved to be the chromosomal breakpoint and delivered a candidate oncogene, *bcl-2*, at 18q21.[7-9]

Bcl-2 represses programmed cell death

Whether the newly discovered genes found at chromosomal breakpoints could be shown to be transforming remained a major question. To assess whether bcl-2 might be involved in a growth factor pathway, vectors overproducing bcl-2 were introduced into a variety of interleukin (IL) dependent cell lines. Such lines were examined to determine if bcl-2 would spare the need for a specific ligand/receptor interaction. However, no long-term growth factor independent cell lines emerged following overexpression of bcl-2 and a more novel effect was noted that helped define a physiologic role for bcl-2. Bcl-2 conferred a death sparing effect to certain haematopoietic cell lines following growth factor withdrawal.[10-12] This was noted in the IL-3 dependent early haematopoietic cell lines although the effect was not restricted to the IL-3/IL-3 receptor signal transduction pathway in that GM-CSF and IL-4 deprived cells displayed a similar response. Yet, bcl-2 enhanced cell survival was not universal as IL-2 dependent T cell lines showed no consistent effect upon factor withdrawal. Even these early studies indicated a selectivity to bcl-2's interference with cell death.

Bcl-2 protein distribution in adult and embryonic tissues

Bcl-2 protein displays a very restricted topographic distribution within mature tissues characterized by apoptotic cell death.[13] One of the most dramatic examples is provided by 2° germinal centers. Immunohistochemical assessment revealed that the follicular mantle, composed of long-lived recirculating IgM/IgD B cells, possessed abundant bcl-2.[13,14] Bcl-2 protein was absent from the dark zone of proliferating centroblasts and from the portion of the light zone where centrocytes are dying by apoptosis. Yet, bcl-2 expression returns in B cells in the more apical portion of the light zone where B cells are selected for survival.

An initial survey of tissues argued strongly that bcl-2 had roles beyond B cells. The thymus has distinct cortical and medullary regions that possess thymocytes at serial stages of maturation. Bcl-2 was present in the surviving $CD4^+$ or $CD8^+$ mature thymocytes of the medulla. The majority of $CD4^+8^+$ immature cortical thymocytes, most of which die by apoptosis, displayed no bcl-2.[13-15] All haematopoietic lineages that derive from a renewing stem cell also display bcl-2. While present in the precursor cells it is absent in their most differentiated and terminal progeny. Another category of tissues that express bcl-2 is glandular epithelium that undergoes hyperplasia or involution, usually in response to hormonal stimuli or growth factors. In organized epithelium, bcl-2 is restricted to stem cell and proliferation zones. Bcl-2 is present in the lower crypts of the intestine and the basal layer of epidermis shows it. Bcl-2 may be required to save the progenitor and long-lived cells in such lineages.

Within the embryo, bcl-2 is expressed in most organs examined (D.J. Veis, S.J. Korsmeyer, submitted). Bcl-2 is expressed in cells within the digital zones of the developing limb bud, but not in the interdigital zones of cell death. Bcl-2 is especially prominent in the nervous system, both in the proliferating ventricular layer and in post-mitotic populations of the cortical plate, cerebellum, hippocampus, and spinal cord (D.E. Merry *et al*, submitted). Bcl-2 expression does not always mirror

recognized patterns of cell death. Bcl-2 expression in the CNS declines with aging. In the regeneration competent peripheral nervous sytem, neurons and supporting cells of sympathetic and sensory ganglia retain substantial bcl-2 protein throughout life. Developmental patterns of *bcl-2* expression suggest that the susceptibility to cell death is much more widespread in the embryo than appreciated, or that bcl-2 has a role beyond the regulation of cell death.

Gain of function transgenic mice satisfy a molecular Koch's postulate

A stringent test of a gene's oncogenic capacity is to place it into the germline of mice to observe its effects during the development of an entire organism. Transgenic mice bearing a *bcl-2* Ig minigene initially displayed a polyclonal follicular lymphoproliferation that selectively expanded a small resting IgM/IgD B cell population. Cell cycle analysis confirmed that ~97% of the expanded B cells reside in G0/G1. These recirculating B cells accumulate because of an extended survival rather than an increased proliferation.[16,17] Over time these transgenics progress from indolent follicular hyperplasia to diffuse large cell immunoblastic lymphoma.[18] A long latency period and progression from polyclonal hyperplasia to monoclonal high-grade malignancy is an indictment of secondary genetic abnormalities. Approximately half of the high grade tumours possess a c-*myc* translocation involving an Ig H chain locus. These tumour cells have complemented an inherent survival advantage (*bcl-2*) with a gene that promotes proliferation (*myc*). When *bcl-2* transgenic mice were mated to *myc* transgenic mice a rapidly emerging undifferentiated haematopoietic leukaemia occurred providing further testimony for the potent synergy of this particular oncogene combination.[19] In addition to promoting cell cycle progression myc has been shown to promote apoptosis.[20] Thus, the overexpression of *myc* may specifically benefit from bcl-2's ability to block apoptosis. Finally, the Bcl-2-Ig mice document the prospective importance of the t(14;18) in setting the stage for tumour progression and lymphomagenesis.

To assess the role of bcl-2 in T cell development transgenic mice were generated in which bcl-2 was redirected to the immature T cells in the cortex of the thymus.[21] The idea was to place this particular antidote to programmed death proximal to positive and negative selection. A separate transgenic model also overexpressed bcl-2 in the thymus and displayed similar effects.[22] The introduction of bcl-2 into the normally vulnerable cortical thymocytes protected them from a wide variety of apoptotic stimuli including glucocorticoids, radiation and anti-CD3 treatment. Moreover, bcl-2 altered T cell maturation increasing a distinct subpopulation of cells that had intermediate levels of TCR, so called TCR/CD3^{Med} cells and promoting maturation along a CD8 pathway. Despite these profound alterations by bcl-2, negative selection still occurred to eliminate self reactive T cells. One interpretation of these transgenic models is that the thymus has multiple death pathways.

Bcl-2's subcellular localization may provide a clue to its function

Bcl-2 has a C-terminal hydrophobic region that functions as a signal-anchor sequence responsible for the integral membrane position of the 25 kDa Bcl-2α product.[23] *In vitro* targeting studies indicate that while it can target microsomes, it selectively integrates into the outer membrane of mitochondria with its NH_2-terminus facing the cytosol. In addition bcl-2 has been localized to other membranes including endoplasmic reticulum and nuclear membrane.[24-26] The majority of bcl-2 within lymphocytes localizes to mitochondria providing a vantage point from which to address bcl-2's potential metabolic role in opposing cell death.

Bcl-2 regulates an anti-oxidant pathway

Bcl-2's ability to block γ-irradiation-induced cell death[21,22] is notable in that ionizing radiation produces hydroxyl radicals ($OH^{\cdot}$) in aqueous solution by radiolytic attack on H_2O.[27] Bcl-2 is localized to the intracellular sites of oxygen free radical generation including mitochondria, endoplasmic reticulum and nuclear membranes. Many of the effects of oxygen free radicals including DNA strand breaks and membrane blebbing match the hallmark features of apoptosis, prompting an examination of bcl-2's ability to counter other oxidative cell deaths. Bcl-2 protected cells from the lethal effect of H_2O_2 in a dose dependent manner, a death that resembles apoptosis morphologically. Bcl-2 also interfered with cell death induced by menadione, a quinone compound which undergoes redox cycles intracellularly to generate superoxide, O_2^-.[28] Importantly bcl-2

could not prevent the cyanide resistant oxidative burst generated by menadione. Moreover, oximetry studies in two separate model systems of apoptosis revealed no evidence for any increase in cyanide resistant oxygen consumption reflective of a burst in the production of reactive oxygen species. Instead, an oxidation sensitive fluorescent probe, DCFH-DA, indicated that generation of endogenous peroxides continued at an inherent rate before and after a death signal. Moreover, overexpression of bcl-2 did not alter the efficiency of normal electron transport nor the production of peroxides.[28] Bcl-2 functions to completely suppress vital damage to cells including lipid membrane peroxidation. Consistent with this finding, overexpression of glutathione peroxidase (GSHPx) but not manganese superoxide dismutase (MnSOD) repressed apoptotic cell death.[27] GSHPx is a known inhibitor of lipid peroxidation. What are the important reactive oxygen species (ROS) in programmed cell death (Figure 2)? These data suggest that peroxides are an important ROS perhaps reflecting the diffusibility of H_2O_2 or its proclivity for conversion to the most highly reactive $OH^{\cdot}$ radical (Figure 2). Bcl-2 appears to block this pathway between peroxide generation and lipid membrane peroxidation.[28] Precisely how bcl-2 performs this role remains a challenge.

Bcl-2 deficient mice argue that bcl-2 is a death repressor molecule functioning in an anti-oxidant pathway

Gene targeting in embryonic stem (ES) cells is a powerful technology to disrupt an allele and create a loss of function whole animal model.[29] A deletional vector and double drug selection was utilized to disrupt the *bcl-2* locus and mice obtained that transmitted the homologously recombined allele to germline.[30] Bcl-2 −/− mice complete embryonic development and appear normal for the first week. They then display growth retardation, small external ears, immature facial features and early mortality post-natally. Haematopoiesis including B and T cell differentiation is initially normal. Bcl-2 is not absolutely required for the development of those lineages. Thus, Bcl-2 is an example in which lessons learned from loss-of-function models are not identical to those from gain-of-function models.

Overtime bcl-2 −/− mice demonstrate fulminant apoptosis of the thymus and spleen.[30] Ill mice show a dramatic decrease in lymphocyte counts. Marked apoptotic death occurred in some lymphoid organs until only fibrous tissue remained. Of importance, bcl-2 −/− thymocytes require an apoptotic stimulus to manifest their predisposition to massive cell death. This finding and an expanding family of bcl-2 related molecules, bcl-x,[31] Mcl-1[32] and A1,[33] may provide a redundancy in death repressor activity and account for the development of bcl-2 −/− animals. This may argue that bcl-2 has a more unique role in maintaining homeostasis in adult tissues.[30]

Despite the widespread expression of Bcl-2 during embryonic development most organs were histologically normal in bcl-2 −/− mice. However, bcl-2 −/− mice develop severe polycystic kidney disease (PKD) characterized by dilated proximal and distal tubular segments and hyperproliferation of epithelium and interstitium. Several metabolic toxins induce PKD which have been shown to inhibit peroxide detoxification. These observations suggest a biochemical link between drug induced PKD and that seen in bcl-2 −/− mice.

Hair growth in the mouse is cyclical. Bcl-2 −/− mice have initially normal black and agouti hair pigmentation. However, with the second hair follicle cycle bcl-2 −/− mice turn grey. The coat colour change is homogeneous and each hair retains some melanin. This may favour a primary biochemical effect on melanin synthesis rather than melanocyte death. A key intermediate in melanin synthesis, DOPA-quinone, is an extremely reactive compound that undergoes cyclization and oxidative polymerization to form the insoluble heteropolymers of eumelanin. A process in which the production of light and dark melanin are regulated by cellular redox

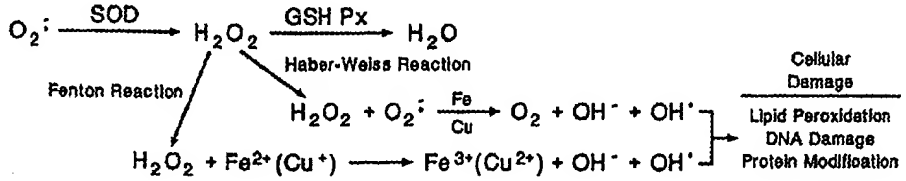

Figure 2. Schematic representation of some major pathways of oxygen free radical generation and detoxification within cells in programmed cell death.

potential. Changes in the metabolism of peroxides and free radicals could impact both melanin synthesis and cellular survival.

Bcl-2/bax: a cell autonomous rheostat controls cell death

Since bcl-2 lacked any conserved functional motifs it was possible that it comprised a member of a multicomponent complex. Immunoprecipitation revealed that bcl-2 heterodimerizes *in vivo* with a 21 kDa partner, bax (bcl-2 associated X protein).[34] Of note, was the unanticipated finding that bax shared homology with bcl-2 clustered within two highly conserved domains I and II. These motifs define a family including *bcl-x*, *mcl-1* and *A1*. These genes are likely to be sequential members in a single death pathway or regulators of parallel pathways. Given the heterodimerization of family members, it was possible that *bax* worked in concert with *bcl-2* to repress death or that this represented 'hand to hand' combat in which *bax* promoted death while *bcl-2* opposed it. Overexpression studies indicated that excess bax countered bcl-2 and accelerated apoptotic cell death, but only following a death signal.[34] Of note, the bcl-2/bax association exists in cells prior to a death stimulus and is not markedly altered in the early death process. When bcl-2 is in excess bcl-2 homodimers dominate and cells are protected. When bax is in excess bax homodimers dominate and cells are susceptible to apoptosis (Figure 3). A number of biologic systems indicate that cells vary during development in their inherent sensitivity or resistance to a given death stimulus. The ratio of bcl-2/bax represents a cell autonomous rheostat that pre-determines a cell's life or death response to an apoptotic stimulus. As a testable model, perhaps such protein/protein interactions could serve to focus as well as regulate an anti-oxidant pathway at the selective sites of ROS generation.

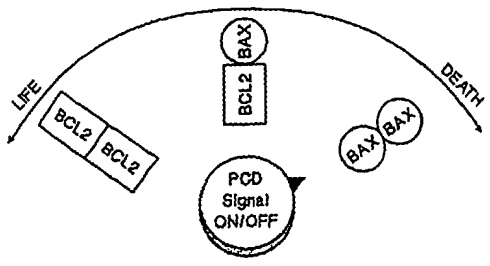

Figure 3. A pre-set rheostat of the ratio of bcl-2/bax determines the life or death response of a cell following a programmed cell death (PCD) signal.

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